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Standardization of Nucleic Acid Tests

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1 **Standardization of nucleic acid amplification tests: the approach of the World Health**

2 **Organization**

3

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15

16 **ABSTRACT**

17 The first World Health Organization (WHO) International Standards (ISs) for nucleic acid
18 amplification techniques (NAT or NAAT) were established two decades ago with the initial
19 focus on blood screening for three major viral targets - hepatitis C virus, hepatitis B virus and
20 human immunodeficiency virus type 1. These reference materials have subsequently found
21 utility in the diagnosis and monitoring of a wide range of infectious diseases in clinical
22 microbiology laboratories worldwide. WHO collaborating centers develop ISs and coordinate
23 international studies for their evaluation. The WHO Expert Committee on Biological
24 Standardization is responsible for the endorsement of new standardization projects as well as
25 establishment of new and replacement ISs. Potencies of ISs are defined in “international
26 units” (IU), and the reporting in IU by assays calibrated with an IS (or secondary standards
27 traceable to the IS) facilitates comparability of results between different assays and
28 determination of assay parameters such as analytical sensitivities.

29
30 **INTRODUCTION**

31 Nucleic acid amplification technology (NAT or NAAT) has become a staple in both the
32 clinical microbiology laboratory and in blood screening centers for the detection of microbial
33 pathogens, particularly viruses. This was not the case more than two decades ago with the
34 transmission of hepatitis B/C viruses (HBV and HCV) and human immunodeficiency virus
35 type 1 (HIV-1) to recipients of therapeutic plasma derivatives or blood components, when it
36 was realized that closing the serological window using NAT testing improved blood safety. In
37 the following years, considerable effort was invested in the implementation of NAT screening
38 for blood and plasma donors and introducing this technology for diagnostic testing in clinical
39 microbiology laboratories using both commercial as well as laboratory developed tests
40 (LDTs). However, assay sensitivities and specificities varied widely between laboratories,
41 contamination by amplicons was problematic and assays lacked standardization. During this

42 time, the World Health Organization (WHO), as the global institution for setting standards for
43 health systems, was requested to establish internationally accepted reference materials, e.g.
44 International Standards (ISs), for NAT assays. The ISs are measurement standards with a
45 defined concentration of a specific analyte that enable the comparison of results between
46 different assays and different laboratories. These reference materials were initially prepared
47 from viremic plasma donations (reflecting the type of sample being tested) and subsequently
48 freeze dried. The complex nature of donor and clinical samples, such as plasma or sera, means
49 that nucleic acid measurement of a specific pathogen cannot be determined by physico-
50 chemical methods. Before nucleic acid concentrations can be determined, samples must be
51 extracted and undergo *in vitro* amplification and detection; therefore results cannot simply be
52 reported in International System of Units (SI)-related units such as kilograms or moles. For
53 WHO ISs representing complex biological materials, the WHO took the approach of adopting
54 the International Unit (IU); the IU has been used to define potencies of all ISs for NAT-based
55 assays.

56 In this review, we discuss the steps involved in prioritization and in the preparation and
57 characterization of WHO ISs, their establishment, replacement and realization of their value
58 in harmonizing results between different assays and different laboratories.

60 **SETTING PRIORITIES FOR NAT STANDARDIZATION**

61 An international working group Standardization of Genomic Amplification Techniques
62 (SoGAT) was established in 1995, on behalf of the WHO, which has since been coordinated
63 by the National Institute for Biological Standards and Control (NIBSC; United Kingdom).
64 Initially, the focus was to standardize NAT assays for blood-borne pathogens important in the
65 field of blood safety; however, standardization was also essential in the diagnosis and
66 monitoring of infectious diseases in the clinical setting. WHO ISs for pathogens such as HCV,

67 HBV and HIV-1 have been widely used in microbiology laboratories as well and new
68 standards have been prepared for increasing numbers of clinically important pathogens.
69 The first WHO IS for NAT assays established in 1997 was HCV (1), this was followed by
70 hepatitis B virus (HBV) and HIV-1 in 1999 (2, 3). Subsequently, ISs have been established
71 for other blood-borne viruses including parvovirus B19 (B19V), hepatitis A virus (HAV),
72 HIV-2, hepatitis E virus (HEV) and hepatitis D virus (HDV) (4-8) as well as human
73 cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (9, 10). Several of these standards,
74 like those for HCV, HBV and HIV-1, have been essential for introducing regulatory
75 requirements for testing of blood and plasma donations as well as being used by clinical
76 microbiology laboratories for determination of viral loads. In the field of transplantation, ISs
77 have been prepared for CMV, EBV, BK virus (BKV), JC virus (JCV) as well as human
78 herpesvirus type 6b (HHV6b) (9-13). Other ISs established include ones for the parasites
79 *Plasmodium falciparum* and *Toxoplasma gondii* (14, 15) as well as a standard for
80 *Mycoplasma* species (16). More recently, emerging diseases have been addressed with the
81 establishment of ISs for Zika virus (ZIKV) and chikungunya virus (CHIKV) (17, 18). Slightly
82 different types of WHO standards, termed reference reagents have been prepared for Ebola
83 virus (19) and the four different dengue virus serotypes (20). Although initially developed for
84 vaccine studies, ISs have been prepared for human papillomavirus type 16 and 18 (21), in this
85 case, based on plasmids representing the viral genomes due to lack of native or cultured
86 source materials. Current WHO ISs and reference reagents for NAT are shown in Table 1.
87 The SoGAT group has met at least annually since it was established, collectively identifying
88 priority pathogens where there is a need for NAT-standardization and coordinating
89 international studies to develop and evaluate these materials. The need for specific standards
90 is determined through discussions with the scientific and medical community worldwide
91 through the SoGAT forum, through WHO programs in disease areas such as malaria and
92 tuberculosis, with input from manufacturers of *in vitro* diagnostic devices (IVDs) and by the

93 three official WHO collaborating centers in the fields of blood and IVDs: NIBSC, the Paul-
94 Ehrlich-Institut (PEI, Germany) and the U.S. Food and Drug Administration, Center for
95 Biologics Evaluation and Research (FDA/ CBER, USA). The SoGAT meetings allow for the
96 discussion of results from international collaborative studies prior to submission and review
97 by the WHO Expert Committee on Biological Standardization (ECBS). The ECBS plays a
98 formal role in the establishment of ISs and related reference materials, and committee
99 members are scientific experts from national control agencies, research institutes, academia,
100 public health bodies and the pharmaceutical industry. All new proposed international
101 standardization projects are subject to review by the ECBS before endorsement.
102 Occasionally, special topics have been discussed at extraordinary SoGAT meetings; examples
103 include addressing the problems with detection of different genotypes of B19V and how to
104 improve standardization (22).

106 **TYPES OF WHO REFERENCE MATERIALS**

107 *International Standards (ISs) and their role*

108 ISs are measurement standards and are assigned an internationally agreed unitage in IU (23).
109 The potencies of ISs are determined by consensus means through international collaborative
110 studies, using a range of methods typically in routine use by participating laboratories. In the
111 case of NAT assays, potencies are determined by a combination of end-point dilution analysis
112 for qualitative assays and, for example, by “copy numbers” or “genome equivalents” for
113 quantitative assays. Although the IU is arbitrary in theory, in practice, it corresponds to the
114 mean overall potency (“NAT-detectable units”) reported by participating laboratories.
115 Adoption of the IU also avoids the issue of copy number, the definition of which is assay-
116 dependent and which also implies, misleadingly, that material is traceable to an SI unit.
117 Repeatedly, during studies to evaluate new ISs, quantitative reporting of concentrations of
118 samples in copy numbers typically varies over several orders of magnitude. This demonstrates

119 that copy number is not a robust measure that can be compared readily between laboratories;
120 the use of the IU allows better comparison of results.

121 WHO ISs are considered as the highest order, international conventional calibrators in
122 accordance with ISO 17511:2003 (*In vitro* diagnostic medical devices - Measurement of
123 quantities in biological samples - Metrological traceability of values assigned to calibrators
124 and control materials) (24). The principal use of ISs is in the calibration of secondary
125 standards (Figure 1), traceable in IU and for evaluation of critical assay parameters such as
126 analytical sensitivities and quantification range, including upper and lower limits of
127 quantification. The preparation and calibration of secondary standards is described in detail
128 elsewhere (25). Uncertainty values are not assigned to WHO ISs, since the IU is an arbitrary
129 unit and variance is associated with that of the vial content.

130 In Europe, the new Regulation on *in vitro* diagnostic medical devices (CE-IVDs) stipulates
131 the design requirements for calibration of assays using “reference materials of a higher
132 metrological order” (26). Furthermore, the Regulation requires metrological traceability of
133 values assigned to calibrators and control materials using “reference materials...of higher
134 order” which should be communicated to the user. In addition, the “Common Technical
135 Specifications” state that WHO ISs should be included in the performance evaluation and the
136 reporting of test results in IU for “high risk” IVDs (e.g. for quantitation of HIV-1, HBV, or
137 HCV) (27). Furthermore, regulatory requirements for testing of biologics may define minimal
138 sensitivity for suitable assays based on WHO ISs. Examples are national requirements for
139 blood screening markers (e.g. HIV-1 RNA, HCV RNA in Germany) or European regulation
140 of plasma derivatives (e.g. HCV RNA in manufacturing plasma pools).

141 Representatives of the US FDA/CBER participate on a regular basis in the international
142 standardization efforts undertaken by WHO. In contrast to the EU, there is no legal

143 requirement in the US to use WHO ISs for assay calibration; however, panel members used
144 by FDA/CBER for lot release of NAT tests have been calibrated against WHO ISs (28, 29).
145 When an IS is established for the first time, it is designated the 1st IS, upon its replacement it
146 is termed the 2nd IS, the 3rd IS and so on and with each subsequent standard replacing its
147 predecessor as the highest order reference standard. Replacement of ISs is discussed in more
148 detail below.

149 ***Reference reagents and international reference panels***

150 In addition to WHO ISs, there are other types of standards established by the WHO ECBS,
151 these include Reference Reagents (RRs) as well as International Reference Panels (IRPs).
152 Both RRs and IRPs are prepared and evaluated using principles similar to WHO ISs.

153 The IRPs consist of different genotypes or important strains of pathogens with diverse global
154 distribution; examples of such panels include HIV, HBV, B19V and HEV (Table 2) (30-35).
155 The role of IRPs is to help ensure consistent detection of pathogen variants, particularly when
156 being used for assay validation purposes. They have been important tools for improvement in
157 assay performance where detection of specific variants has been sub-optimal. Usually, no
158 unitage is assigned to members of IRPs. However, the data on assay performance are included
159 in the collaborative study reports published on the WHO website, providing a range of
160 potencies reported for individual panel members.

161 In the case of RRs, these are usually interim standards with a unitage defined in units rather
162 than IU. Upon further characterization, RRs may be established as ISs and the unitage defined
163 in IU. Examples of RRs include NAT standards for Ebola virus, established in response to the
164 Ebola crisis in 2014, and based upon recombinant lentivirus vectors to avoid biosafety issues
165 (19). More recently, four RRs have been established for dengue types 1-4; because of the

166 genetic differences between the types it was not possible to select a single strain as an IS,
167 consequently each type has a separate unitage (20).

168

169 **PREPARATION AND ESTABLISHMENT OF WHO REFERENCE MATERIALS**

170 *Characterization and preparation of candidate standards*

171 The processes involved from the identification of the scientific need to develop a standard
172 through establishment and ultimately its replacement are shown in Figure 2. The procedure to
173 establish WHO standards is extremely rigorous (23) and undertaken by one of the three WHO
174 Collaborating Centers on behalf of the WHO.

175 The development of a new standard starts with the identification and preparation of a suitable
176 stock material which may either be viremic plasma – for example for HCV, HBV and HEV or
177 parasitemic whole blood (*Plasmodium falciparum*) (14), or pathogens propagated in culture.
178 More rarely, animals have been used as alternative starting materials where sources of native
179 materials are unavailable or not of insufficiently high titer, and example of this is the
180 propagation of *Toxoplasma gondii* tachyzoites in mice (15). HPV ISs have been based on the
181 preparation of plasmid DNAs diluted in human genomic DNA (21). An estimate is made of
182 the concentration of the stock material and identity testing is performed e.g. by sequence
183 analysis, and where material has been obtained from blood or plasma, donations are screened
184 to ensure the absence of other blood-borne pathogens other than the target in question. Strains
185 are selected to reflect those with widespread distribution and global importance whenever
186 possible. Occasionally, materials may be inactivated depending on feasibility combined with
187 biosafety concerns; such procedures should be validated, however, this may not be possible
188 for some pathogens where suitable cell culture systems are not available. To facilitate
189 distribution worldwide, WHO standards are usually lyophilized. Therefore, formulation is an
190 important factor to consider and this is fairly straightforward where viremic plasma is used

191 and the standards will be further diluted in this matrix when used in the recipient laboratories.
192 However, where testing of certain pathogens can be performed on different types of matrices,
193 e.g. whole blood, urine, cerebrospinal fluid (CSF) as well as plasma, cultured viral and
194 microbial strains have been formulated in solutions containing excipients (buffers, sugars,
195 stabilizers etc.) that allow further dilution of the standard into the appropriate type of matrix.
196 The final formulation should not cause any interference with the NAT assays, e.g. decrease in
197 extraction efficiency or inhibition of amplification.

198 When the bulk standard preparation is dispensed into either vials or ampoules, the coefficient
199 of variation of the filled volume is determined. Several thousand vials/ampoules are usually
200 prepared. After lyophilization, the ampoules or vials are back-filled with nitrogen and the
201 homogeneity of the lyophilized material is determined, sampling across the batch. Testing is
202 performed for residual moisture and oxygen which may impact product stability and
203 accelerated (at higher temperatures) and real-time stability is determined to ascertain that the
204 reference material can be shipped at ambient temperatures worldwide, without loss of potency
205 under normal storage temperatures (typically -20°C) over the life of the IS.

206 ***Commutability***

207 Commutability is a property of a reference material demonstrated by the closeness of
208 agreement between the results obtained for the reference material and the results obtained for
209 clinical specimens, when comparatively tested in different assays (36, 37). In other words, in
210 order to be suitable as an assay calibrator, the reference material should not behave differently
211 compared to clinical specimens. Commutability is demonstrated by testing the different
212 materials (reference material, clinical specimens) in multiple assays. ISs are designed to
213 reflect as closely as possible the specimens tested in routine diagnosis or blood screening. For
214 example, human plasma or sera are very common types of sample matrices tested in blood
215 screening and clinical laboratories and several ISs are derived from viremic donations or

216 contain culture-derived virus diluted in plasma. In addition, the strain of pathogen (i.e. the
217 analyte) used for the IS is usually selected to represent the most commonly circulating variant.
218 Commutability is an important precondition for the ability of the calibrant to harmonize
219 different assays, and is addressed by inclusion of clinical specimens, as far as possible, in the
220 international collaborative study. The impact of different extraction systems (reagents,
221 equipment) on the extraction efficiencies for different matrices is another factor to be
222 addressed in commutability studies. In the case of CMV, non-commutability of the IS has
223 been demonstrated for some assays (38). Commutability, in the case of CMV, is particularly
224 complex and affected by features such as the physical form of viral DNA in the IS (virion-
225 associated DNA) compared to that found in transplant patients which is highly fragmented
226 (39, 40). Furthermore, during amplification/detection reactions, amplicon length impacts viral
227 load determinations (40). With the development of additional IS for clinical pathogens the
228 challenge of commutability becomes even more complex with quantitative values reported for
229 multiple types of sample matrices, including urine, CSF and stool. In the case of CSF, it is a
230 matrix with a low protein content which is difficult to obtain in large volumes, and is not easy
231 to evaluate in collaborative studies or in formal commutability investigations. Stool is another
232 challenging sample type where the matrix contains inhibitors and the sample extraction is not
233 well standardized.

234 ***International collaborative studies***

235 Candidate ISs, RRs and IPRS are evaluated in international collaborative studies. Participants
236 volunteering to take part in these studies include blood centers, reference laboratories, clinical
237 microbiology laboratories, manufacturers of diagnostics kits and medicinal products as well
238 as regulatory organizations. Typically 15 to 25 laboratories will be involved in such a study.
239 The assays included in the studies are ones used throughout the world, and include
240 commercially available tests as well as LDTs. The studies investigate potency of the
241 candidate materials, clinical comparator samples as well as related reference materials and

242 calibrators; potencies are determined using qualitative or quantitative assays as described
243 above. One of the major aims of each study is to provide a basis for assignment of unitage to
244 the standard; the unitage assignment is usually based on the combined mean potency for all
245 the assays included in the study. Expressing results of the study samples against the candidate
246 IS can greatly reduce variation in the measured potencies reported by participants, and the
247 harmonization effect (see below) is an important factor reviewed by the ECBS to demonstrate
248 the utility of a new IS. The studies themselves allow a head-to-head comparison of assays
249 used throughout the world and provide information on sensitivity (based on end-point analysis
250 of qualitative assays) as well as variability in quantification.

251 Statistical analysis of the study data forms the basis for the final report which includes a
252 proposal for the unitage for the IS. Participants are requested to comment on the report and
253 asked if they agree with the proposed unitage. The final report is made available on the WHO
254 website for public review ahead of the annual meeting of the ECBS. In the case of IRPs, no
255 unitage is assigned to the panel members; however, details may be included in the report with
256 the range of potencies observed.

257 Subsequent to the establishment of a standard or panel, the custodian laboratory has a
258 responsibility for the storage of each batch under controlled conditions, monitoring of stability
259 and coordinating distribution worldwide.

260

261 **REPLACEMENT OF WHO ISs**

262 Although several thousand vials are prepared for each standard, when they are nearing
263 exhaustion, it is essential to replace the previous preparation. Replacement projects are
264 prioritized by the WHO. An important aspect of replacement of one standard with the next is
265 maintaining the continuity of the IU in order to ensure that tests can be compared over time.
266 Details of the NAT standards which have been replaced are shown in supplementary Table
267 S1. Since it was established in 1997 (1), the HCV IS has been replaced four times (41-44).

268 Replacement ISs have been prepared for HBV (45-47), HAV (48, 49), HIV-1 (50-52) and
269 B19V (53, 54). In each case, replacement preparations have been evaluated in parallel with
270 the previous IS, using either qualitative end-point assays or quantitative assays (within the
271 linear range) and covering appropriate dilutions. With each subsequent IS, the possibility
272 exists for drift in the IU; this may be exacerbated by issues with assay features included in
273 collaborative studies, such as primer/probe mismatches affecting quantification, and
274 emphasizes the need for good characterization of starting materials. An example is the study
275 to establish the 3rd IS for B19V (54) where the new B19V viremic plasma donation used for
276 the 3rd IS was under-quantified by the COBAS TaqScreen DPX test, probably due to a
277 mismatch between the primers/probe and the sequence of the ISs (55), impacting the assigned
278 unitage.

279

280 **ASSAY HARMONIZATION USING WHO ISs**

281 ***Relative potencies***

282 During the establishment of WHO ISs, one of the criteria for acceptance of a new standard is
283 the demonstration that when results of testing are expressed relative to the candidate IS, an
284 improvement is seen in the agreement observed between assays and laboratories.

285 An example of this is shown in Figure S1. A HEV sample, included in the collaborative study
286 to establish the HEV IS, was evaluated using a mixture of qualitative and quantitative NAT
287 assays – the reported potencies are shown in the upper panel showing a wide variation in titres
288 over several orders of magnitude. By expressing these potencies against the WHO IS (PEI
289 code number 6329/10) the agreement between laboratories is markedly improved with
290 variation being reduced to ~ 1 log₁₀ and a typical reduction in the associated standard
291 deviation (SD).

292 ***External Quality Assessment Programs***

293 External quality assessment (EQA)/proficiency testing (PT) programs can be very helpful in
294 generating data on the implementation of WHO ISs by participating laboratories in a large
295 number of countries. In some cases, WHO ISs have been included directly in EQA studies.
296 For example, the 1st IS for ZIKV was made available by the WHO in July 2016 prior to
297 formal establishment by the ECBS and was introduced as a consequence of the Public Health
298 Emergency of International Concern (56). The 1st ZIKV IS has been included in all the ZIKV
299 EQA/PT programs provided by Quality Control for Molecular Diagnostics (QCMD) since
300 2016 (57).

301 Data analysis from QCMD EQA/PT schemes demonstrate that where an IS has been
302 established for a specific target pathogen the observed variation (SD) based on the geometric
303 mean of the log₁₀ viral load results, are noticeably smaller (Table S2). This observation is
304 based on results reported in IU/mL on duplicate panel members. In contrast, for pathogen
305 targets where an IS has only recently been established or where there is no IS and reporting of
306 results is often in different types of unit, the SDs are much greater (Table S2). In addition,
307 where there is a known clinical need for pathogen quantitation then the IS and IU/mL are
308 more readily accepted.

309 In the case of CMV, for example, in early EQA/PT studies done prior to 2004, the majority of
310 assays performed by laboratories participating in the CMV EQA program were qualitative
311 (Figure S2). For quantitative assays performed prior to the establishment of the 1st CMV IS in
312 2010 (9), laboratories reported results in either copies/mL or other units of measurement such
313 as genome equivalents/mL as observed through the data reported in international EQA/PT
314 schemes. Over the last 8 years the number of laboratories reporting in IU/ml has increased
315 significant from 0 to 50% of the datasets returned within the annual international EQA/PT
316 schemes run by QCMD (Figure S3). For CMV viral load testing, the increase in reporting in
317 IU correlates with an increase in the use of commercial assays used by participants in the
318 QCMD studies (Figure S4). In a recently published EQA study, evaluating results reported in

319 IU/mL, the variation between results was lower when compared to those reported in
320 copies/mL demonstrating the use of the CMV WHO IS improves the reproducibility and
321 comparability of CMV viral load results across laboratories (58). Consequently, the recently
322 revised International guidelines on the management of CMV in solid organ transplantation
323 recommend that all results should be reported as IU/mL (59). More significant improvements
324 in results have been reported for EBV when the IS has been used (60).

325

326 **PRE-QUALIFICATION OF *IN VITRO* DIAGNOSTIC DEVICES**

327 International reference preparations play an important role in the WHO prequalification
328 program for IVDs. In this program, IVDs targeting low- and middle-income countries (LMIC)
329 are independently assessed by WHO since LMIC themselves rarely have the regulatory
330 capacity to assess the quality and suitability of IVDs offered to the national market. In WHO
331 prequalification studies, ISs may be used for comparative evaluation of essential assay
332 features such as sensitivity, limit of detection or range of quantitation. Furthermore, IRPs
333 covering different variants (e.g. genotypes, recombinants) are important for the detection of
334 strains more prevalent in certain regions. The outcome of performance evaluation studies
335 initiated on behalf of the WHO prequalification program for IVDs is published together with
336 a list of IVDs deemed suitable by WHO for the intended purpose.

337

338 **STRATEGIC ADVISORY GROUP OF EXPERTS ON *IN VITRO* DIAGNOSTICS** 339 **(SAGE IVD)**

340 In 2017, the WHO established the Strategic Advisory Group of Experts on *In Vitro*
341 Diagnostics (SAGE IVD). SAGE IVD recently published the first model list of essential
342 diagnostics, including several NAT assays for markers including HBV, HCV, HIV,
343 *Mycobacterium tuberculosis* and HPV (61). The elaboration of the list is aimed to improve

344 access to IVDs which are estimated essential in a given health system. The ultimate goal is
345 strengthening of health systems and the availability of universal health coverage. This is akin
346 to the WHO essential medicines list which includes those medicines which are deemed
347 indispensable in a health care system.

348

349 **STANDARDS CURRENTLY UNDER DEVELOPMENT**

350 Standards currently under development are shown in the supplementary Table S3 and include
351 viral and parasitic markers as well as a standard for *M. tuberculosis* reflecting the global
352 burden of disease and the increasing use of molecular testing for this pathogen.

353

354 **CONCLUSIONS**

355 Significant progress has been made in NAT standardization over the past two decades in the
356 context of screening for blood-borne markers as well as in clinical diagnostic laboratories.
357 The development of WHO standards and other reference reagents (ISs, RRs and IRPs) has
358 helped in these efforts, also enabling the introduction of regulations for the detection of blood-
359 borne pathogens in the fields of transfusion and blood product safety for markers such as
360 HCV, HBV, HIV, HAV, B19V and more recently HEV by setting thresholds and control
361 concentrations, defined in IUs. For clinical laboratories, for diagnosis and treatment
362 monitoring, HCV, HBV and HIV-1 standards have been important for viral load
363 determinations; in relation to transplantation standards established for CMV, EBV, HEV,
364 BKV, JCV and HHV-6b are used for expression of viral loads in IU. The use of the IU
365 improves agreement and allows comparability of data between laboratories and allows the
366 introduction of regulations in blood screening using NAT and informs clinicians in patient
367 testing and monitoring of therapeutic interventions. International clinical guidelines e.g. for

368 CMV and HEV in the transplant setting, reporting in IU is encouraged further supporting
369 accuracy in viral load reporting and harmonization efforts (59, 62). These efforts are
370 underpinned by the secondary standards and controls traceable in IU as well as calibrated
371 assays.

372 Because of their biological nature, WHO standards control for the entire NAT process –
373 including nucleic acid extraction. Organizations such as the National Institute of Standards
374 Technology in the US, take a different approach and produce “standard reference materials”
375 (SRMs) for a small number of viral markers including a bacterial artificial chromosome
376 (BAC), containing the genome of the CMV Towne strain and a linearized plasmid DNA
377 control for BK virus. These SRMs are added directly to the amplification/detection reaction
378 without undergoing prior extraction and are intended to be used for the calibration of controls
379 and standards. Some organizations provide *in vitro* transcribed RNAs (IVTs), and like the
380 NIST materials these materials do no control for the extraction part of the NAT assay. In a
381 study organized by kit manufacturers, a partial HCV IVT RNA was evaluated in a study
382 comparing amplification methods; however it was not found to perform better than the
383 biological standard (63). During the study to establish the 1st WHO IS for CMV, the candidate
384 standard, based on a clinical strain (Merlin) propagated in cell culture, was evaluated in
385 parallel with BAC containing the entire Merlin genome. Participants added the BAC directly
386 to the amplification reactions. Expression of potencies of other cultured virus preparations
387 against the candidate IS showed marked reduction in variation between laboratories, however,
388 when the results were expressed relative to the BAC no improvement was observed compared
389 to the absolute mean estimates (9). In the study to establish the 1st WHO IS for ZIKV,
390 expression of clinical samples and biological reference materials saw an improvement in
391 agreement of results between laboratories. In the study, two related IVTs were included – one
392 containing several assay target sequences in a single transcript and the second preparation a
393 mixture of the respective individual IVT RNAs. Expressions of the one IVT preparation

394 against the other resulted in harmonization, however, expression of clinical samples or
395 biological reference materials against the IVTs failed to produce any improvement (17).
396 These studies demonstrate the importance of controlling the extraction step in the NAT
397 procedure and emphasizes the advantage of the approach taken by the WHO compared with
398 (bio)-synthetic types of reference material. However, the latter may be easier to replace
399 compared to sourcing, for example, new viremic donations in the case of some of the WHO
400 ISs.

401 Sequence data is available for most WHO ISs, RRs and IRPs (Table 1, supplementary
402 information and Tables S4-S7), sometimes indicating sequence heterogeneities when
403 compared to clinical isolates, e.g. sequence deletions or sequence duplications in culture
404 based materials. Using next generation sequencing data, even subpopulations of sequence
405 variants are being detected, as was reported recently for the ISs BK and JC polyoma viruses
406 (64, 65). Passage of the strains in cell culture resulted in heterogeneous DNA populations, the
407 reason for which is not understood and which could affect some specialized assays (64, 65),
408 although both preparations were shown to successfully harmonize assay performance in the
409 collaborative studies (11, 12) and in independent studies (66). These observations demonstrate
410 the importance in thorough characterization of the starting materials used for standard
411 preparation. Methods such as digital PCR are useful in the characterization process in
412 understanding the relationship between IU and copy number ratios for specific methods as
413 well as for estimating potency during development of new ISs or when no standard exists. In
414 the case of the 1st WHO IS for HAV, the IU:copy number ratio was determined to be 1:14
415 using digital PCR (S. Baylis unpublished data) and the low IU value was a consequence of
416 low sensitivity of assays used by participants in the original collaborative study (5).

417 With the absence of reference methods to define nucleic acid content of microbial pathogens
418 in complex biological matrices, this emphasizes the validity of WHO approach in the

419 development of reference standards and harmonizing NAT assays. However, the challenge for
420 the development of such standard remains meeting the clinical need in a timely manner whilst
421 maintaining rigorous procedures in the establishment process. Adequate commutability of ISs
422 is essential particularly in the clinical setting and will affect treatment of patients and hinder
423 the introduction of clinical practice guidelines. Inclusion of sufficient clinical materials in
424 studies to evaluate commutability remains a problem in terms of volume, transfer agreements
425 and the support of the wider scientific community in these efforts is essential to fully realize
426 the potential of the WHO standardization efforts.

427

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430 over the years.

431 **FOOTNOTES**

432 Details of the reference preparations are available on the WHO website as well as on the
433 respective collaborating centers websites.

434

435 **FIGURE LEGENDS**

436 Figure 1 Hierarchy of standards

437 The relationship between ISs and secondary and tertiary standards is shown together with
438 their uses.

439 Figure 2 Process for the development of WHO ISs, RRs and IRPs

440 The procedure is shown from the identification of a scientific need to develop a standard to its
441 establishment and ultimately its replacement. cIS – candidate International Standard.

442

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Table 1 Current viral and microbial WHO International Standards and Reference Reagents for NAT

International Standards and Reference Reagents for NAT				
Preparation (unitage)	Standard (code number)	Material (accession no.)*	Year of establishment	Reference
BK virus DNA (10,000,000 IU/vial)	1 st International Standard (14/212)	Cultured BK virus, diluted in buffer/human serum albumin/trehalose	2015	11
Chikungunya virus RNA (1,250,000 IU/vial)	1 st International Standard (11785/16)	Cultured and heat inactivated R91064 strain diluted in human plasma (KJ941050).	2017	18
Dengue virus RNA (13,500 units/vial)	1 st Reference Reagent	Cultured and heat inactivated Hawaii strain diluted in human plasma (KM204119).	2016	20
Dengue virus RNA (69,200 units/vial)	1 st Reference Reagent	Cultured and heat inactivated New Guinea C strain diluted in human plasma (KM204118).	2016	20
Dengue virus RNA (23,400 units/vial)	1 st Reference Reagent	Cultured and heat inactivated H87 strain diluted in human plasma (KU050695).	2016	20
Dengue virus RNA (33,900 units/vial)	1 st Reference Reagent	Cultured and heat inactivated H241 strain diluted in human plasma (KR011349).	2016	20
Ebola virus NP-VP35-GP (32,000,000 units/vial)	1 st Reference Reagent (15/222)	Lentiviral vector encoding Ebola genes np-vp35-gp in buffer/human serum albumin/trehalose (KT186367).	2015	19
Ebola virus VP40-L (50,000,000 units/vial)	1 st Reference Reagent (15/224)	Lentiviral vector encoding Ebola vp40-L genes in buffer/human serum albumin/trehalose (KT186368).	2015	19
Epstein Barr virus DNA (5,000,000 IU/vial)	1 st International Standard (09/260)	Cultured EBV B95-8 strain, diluted in buffer/human serum albumin/trehalose (V01555).	2011	10

International Standards and Reference Reagents for NAT				
Preparation (unitage)	Standard (code number)	Material (accession no.)*	Year of establishment	Reference
Hepatitis A virus RNA (15,451 IU/vial)	3 rd International Standard (15/276)	Viremic human plasma (KY003229).	2017	49
Human cytomegalovirus DNA (5,000,000 IU/vial)	1 st International Standard (09/162)	Cultured Merlin strain, diluted in buffer/human serum albumin/trehalose (AY446894).	2010	9
Hepatitis B virus DNA (477,500 IU/vial)	4 th International Standard (10/266)	Viremic human plasma representing HBV genotype A2, HBsAg subtype adw2 (KY003230).	2016	47
Hepatitis C virus RNA (100,000 IU/vial)	5 th International Standard (14/150)	Viremic human plasma representing HCV genotype 1	2015	44
Hepatitis D virus RNA (287,500 IU/ml)	1 st International Standard (7657/12)	Viremic human plasma (HQ005369).	2013	8
Hepatitis E virus RNA (125,000 IU/vial)	1 st International Standard (10/6329)	Viremic human plasma representing HEV genotype 3a (AB630970).	2011	7
HIV-1 RNA (125,893 IU/vial)	4 th International Standard (16/194)	Cultured and heat inactivated subtype B isolate diluted in human plasma (KJ019215).	2017	52
HIV-2 RNA (1,000 IU/vial)	1 st International Standard (08/150)	Cultured and heat inactivated CAM2 strain diluted in human plasma (KU179861).	2009	6
Human Herpes Virus 6B (56,234,132 IU/vial)	1 st International Standard (15/266)	Cultured HHV-6B strain Z-29, diluted in buffer/human serum albumin/trehalose (AF157706).	2017	13
Human papilloma virus type 16 DNA (5,000,000)	1 st International Standard (06/202)	HPV type 16 plasmid DNA diluted in buffer/trehalose (K02718).	2008	21

International Standards and Reference Reagents for NAT				
Preparation (unitage)	Standard (code number)	Material (accession no.)*	Year of establishment	Reference
IU/vial)				
Human papilloma virus type 18 DNA (5,000,000 IU/vial)	1 st International Standard (06/206)	HPV type 18 plasmid DNA diluted in buffer/trehalose (X05015).	2008	21
JC virus DNA (10,000,000 IU/vial)	1 st International Standard (14/114)	Cultured JC virus, diluted in buffer/ human serum albumin/trehalose	2015	12
Mycoplasma DNA (100,000 IU/vial)	1 st International Standard (8293/13)	Cultured <i>Mycoplasma fermentans</i> , in Mycosafe Friis medium	2013	16
Parvovirus B19 DNA (705,000 IU/vial)	3 rd International Standard (12/208)	Viremic human plasma representing B19 genotype 1	2013	54
<i>Plasmodium falciparum</i> DNA (500,000,000 IU/vial)	1 st International Standard (04/176)	Parasitemic human blood	2006	14
<i>Toxoplasma gondii</i> (500,000 IU/vial)	1 st International Standard (10/242)	<i>T. gondii</i> tachyzoites obtained from infected mice, diluted in buffer/trehalose	2014	15
Zika virus RNA (25,000,000 IU/vial)	1 st International Standard (11468/16)	Cultured and heat inactivated PF13/251013-18 strain diluted in stabilizer (KX369547).	2016	17

*Sequences are unavailable for some ISs

Table 2 Current International Reference Panels for NAT (viral markers)

International Reference Panels for NAT				
Panels (No. of members)	Standard (code number)	Material	Year of establishment	Reference
Hepatitis B Virus genotypes (15)	1 st International Reference Panel (5086/08)	Viremic plasma diluted in pooled human plasma; HBV genotypes A-G	2009	33
Hepatitis E virus genotypes (11)	1 st International Reference Panel (8578/13)	Viremic plasma donations and stool samples diluted in pooled human plasma; HEV genotypes 1a, 1e, 2a, 3b, 3c, 3e, 3f/1, 3 ra, 4c, 4g	2015	34
HIV-1 subtypes (10)	1 st International Reference Panel (01/466)	Cultured HIV-1 subtypes A, B, C, D, AE, F, G, AG-GH, N and O diluted in human plasma	2003; replaced in 2012 by 12/224	30
HIV-1 subtypes (10)	2 nd International Reference Panel (12/224)	Cultured and heat inactivated HIV-1 subtypes A, B, C, D, AE, F, G, AG-GH, N and O diluted in human plasma	2012	31
HIV-1 circulating recombinant forms (10)	1 st International Reference Panel (13/214)	Cultured and heat inactivated HIV-1 CRFs and subtype variants diluted in human pooled plasma	2013	32
Parvovirus B19 genotypes (4)	1 st International Reference Panel (09/110; CBER Parvovirus B19 Genotype Panel 1)	Viremic plasma donations diluted in pooled human plasma; B19V genotypes 1a1, 2, 3a and negative plasma control	2009	34

*Sequence details for IRP members are available in supplementary information (text and Tables S4-S7).



